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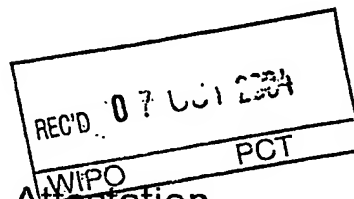
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Patentanmeldung Nr. Patent application No. Demande de brevet n°

03254989.1



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Monofunctional polysialic acids for protein derivatisation and conjugation

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Monofunctional Polysialic Acids for Protein Derivatisation and Conjugation

The present invention relates to derivatives of polysaccharides having at least terminal sialic units, and preferably consisting essentially only of sialic acid units having an aldehyde group for reaction with substrates at the reducing terminal end but not at the non-reducing end. The derivatives are useful for conversion to other reactive derivatives and for conjugation to amine-group containing substrates such as peptides, proteins, drugs, drug delivery systems (e.g. liposomes), viruses, cells, e.g. animal cells, microbes, synthetic polymers etc.

Polysialic acids (PSAs) are naturally occurring unbranched polymers of sialic acid produced by certain bacterial strains and in mammals in certain cells [Roth et. al., 1993]. They can be produced in various degrees of polymerisation from $n =$ about 80 or more sialic acid residues down to $n =$ 2 by limited acid hydrolysis or by digestion with neuraminidases, or by fractionation of the natural, bacterially derived forms of the polymer. The composition of different polysialic acids also varies such that there are homopolymeric forms i.e. the alpha-2,8-linked polysialic acid comprising the capsular polysaccharide of *E. coli* strain K1 and the group-B meningococci, which is also found on the embryonic form of the neuronal cell adhesion molecule (N-CAM). Heteropolymeric forms also exist – such as the alternating alpha-2,8 alpha-2,9 polysialic acid of *E. coli* strain K92 and group C polysaccharides of *N. meningitidis*. Sialic acid may also be found in alternating copolymers with monomers other than sialic acid such as group W135 or group Y of *N. meningitidis*. Polysialic acids have important biological functions including the evasion of the immune and complement systems by pathogenic bacteria and the regulation of glial adhesiveness of immature neurons during foetal development (wherein the polymer has an anti-adhesive function) [Muhlenhoff et. al., 1998; Rutishauser, 1989; Troy, 1990, 1992; Cho and Troy, 1994], although there are no known receptors for polysialic acids in mammals. The alpha-2,8-linked polysialic acid of *E. coli*

strain K1 is also known as 'colominic acid' and is used (in various lengths) to exemplify the present invention.

The alpha-2,8 linked form of polysialic acid, among bacterial polysaccharides, is uniquely non-immunogenic (eliciting neither T-cell or antibody responses in mammalian subjects, even when conjugated to immunogenic carrier proteins) which may reflect its status as a mammalian (as well as a bacterial) polymer. Shorter forms of the polymer (up to n=4) are found on cell-surface gangliosides, which are widely distributed in the body, and are believed to effectively impose and maintain immunological tolerance to polysialic acid. In recent years, the biological properties of polysialic acids, particularly those of the alpha-2,8 linked homopolymeric polysialic acid, have been exploited to modify the pharmacokinetic properties of protein and low molecular weight drug molecules [Gregoriadis, 2001; Jain et. al., 2003; US-A-5846,951; WO-A-0187922]. Polysialic acid derivatisation gives rise to dramatic improvements in circulating half-life for a number of therapeutic proteins including catalase and asparaginase [Fernandes and Gregoriadis, 1996 and 1997], and also allows such proteins to be used in the face of pre-existing antibodies raised as an undesirable (and sometimes inevitable) consequence of prior exposure to the therapeutic protein [Fernandes and Gregoriadis, 2001]. In many respects, the modified properties of polysialylated proteins are comparable to proteins derivatised with polyethylene glycol (PEG). For example, in each case, half-lives are increased, and proteins and peptides are more stable to proteolytic digestion, but retention of biological activity appears to be greater with PSA than with PEG [Hreczuk-Hirst et. al., 2002]. Also, there are questions about the use of PEG with therapeutic agents that have to be administered chronically, since PEG is only very slowly biodegradable [Beranova et.al., 2000] and high molecular weight forms tend to accumulate in the tissues. Likewise there are concerns about the utility of PEG in therapeutic agents that may require high dosages, since accumulation of PEG may lead to toxicity. The alpha- 2,8 linked polysialic acid (PSA) therefore offers an

attractive alternative to PEG – being an immunologically invisible biodegradable polymer which is naturally part of the human body, and which degrades, via tissue neuraminidases, to sialic acid – a non-toxic saccharide.

5 Our group has described, in previous scientific papers and in granted patents, the utility of natural polysialic acids in improving the pharmacokinetic properties of protein therapeutics [Gregoriadis, 2001; Fernandes and Gregoriadis; 1996, 1997, 2001; Gregoriadis et. al., 1993, 1998, 2000; Hreczuk-Hirst et. al., 2002; Mital, 2003; Jain et. al., 2003; US-A-
10 05846,951; WO-A-0187922]. Now, we describe new derivatives of PSAs, which allow new compositions and methods of production of PSA-derivatised proteins (and other forms of therapeutic agent). These new materials and methods are particularly suitable for the production of PSA-derivatised therapeutic agents intended for use in humans and animals, where the
15 chemical and molecular definition of drug entities is of major importance because of the safety requirements of medical ethics and of the regulatory authorities (e.g. FDA, EMEA).

 Methods have been described previously for the attachment of polysaccharides to therapeutic agents such as proteins [Jennings and
20 Lugowski 1981, US-A-5846,951; WO-A-0187922]. Some of these methods depend upon chemical derivatisation of the 'non-reducing' end of the polymer to create a protein-reactive aldehyde moiety (Fig. 1). This is because the reducing end of PSA is only weakly reactive with proteins under the mild conditions necessary to preserve protein conformation and the
25 chemical integrity of PSA and protein during conjugation. The non-reducing end however, since it contains vicinal diols, can be readily (and selectively) oxidised with periodate to yield a mono-aldehyde form, which is much more reactive towards proteins, and which comprises a suitably reactive element for the attachment of proteins via reductive amination and other chemistries.
30 We have described this previously in US-A-5846,951; WO-A-0187922. The reaction is illustrated in figure 1 in which

a) shows the oxidation of colominic acid (alpha-2,8 linked polysialic acid from *E. coli*) with sodium periodate to form a protein-reactive aldehyde at the non-reducing end and

5 b) shows the selective reduction of the Schiff's base with sodium cyanoborohydride to form a stable irreversible covalent bond with the protein amino group

Of the various methods, which have been described to attach polysialic acids to therapeutic agents [US-A-5846,951; WO-A-0187922], none of these involve chemical modification of the reducing end, because of
10 its weak reactivity towards therapeutic proteins. Although theoretically a useful reaction, achievement of acceptable yields of conjugate via reaction of proteins with the hemiketal of the reducing end of the PSA requires reaction times that are not conducive to protein stability. Secondly, reactant concentrations (of polymer excess) are required that may be unattainable or
15 uneconomical. Nevertheless, despite the inefficiency of this reaction, we have observed that it gives rise to unintentional by-products during conjugation reactions intended to produce conjugates with protein via an introduced aldehyde at the (opposite) non-reducing end of the polymer. The potential for such by-products is evident in published studies of catalase,
20 insulin and asparaginase [Fernandes and Gregoriadis; 1996, 1997, 2001; Jain et. al., 2003], where the hemiketal of the natural (chemically unmodified) form of the polymer gives rise to protein conjugates at a low level of efficiency (less than 5% of protein becoming derivatised, see further below in the reference examples, and table1) during reductive amination.
25 The reactivity of the reducing end of colominic acid, though weak towards protein targets, is sufficient to be troublesome in the manufacture of chemically defined conjugates of the kind likely to be preferred by regulatory authorities for therapeutic use in man and animals. Unlike the natural colominic acid polymer, which is weakly monofunctional, the periodate
30 oxidised form of PSA (having an aldehyde at one end and a hemiketal at the other) inevitably gives rise to a complexity of products which seriously

complicate the task of producing a molecularly defined and pharmaceutically acceptable conjugate (Fig 2). Figure 2a is a schematic diagram showing the formation of by-products during polysialylation (original method) Figure 2b is a more detailed schematic diagram showing the formation of by-products during polysialylation (original method), specifically

- i) asymmetric dimer
- ii) linear polymer
- iii) branched polymer
- iv) various more-complex structures

At first sight it would seem a simple matter to purify the intended reaction product away from the various unintended products described in Fig. 2, however, this is by no means straightforward, since the physicochemical characteristics of some of the intended forms (size charge etc.) are remarkably similar, indeed almost identical, to those of the intended form of the product. This would frustrate attempts to purify out the intended species from the reaction mixture by techniques such as ion-exchange chromatography and gel-permeation chromatography (which separate on the basis of charge and size respectively), and would also frustrate many other methods of purification. Now therefore we have solved the problems by developing a new method for conjugation of polysaccharides having sialic acid groups at the reducing terminal to proteins, whereby the weak reactivity of the reducing end can be exploited to beneficial effect, and which avoids the product complexity described in Fig 2(b) using the established method (Fig. 1) of reductive amination of proteins with periodate oxidised natural colominic acid.

Jennings and Lugowski, in US 4,356,170, describe derivatisation of bacterial polysaccharides to proteins via an activated reducing terminal unit involving a preliminary reduction step then an oxidation step. They suggest this approach where the terminal unit is N-acetyl mannosamine, glucose, glucosamine, rhamnose and ribose. There is no suggestion of avoidance of simultaneous reaction at both terminal units.

In the invention there is provided a new process for producing an aldehyde derivative of a polysaccharide in which a polysaccharide starting material having a sialic acid unit at the reducing terminal is subjected to sequential steps of

- 5 a) reduction to reductively open the ring of the reducing terminal sialic acid unit whereby a vicinal diol group is formed; and
- b) selective oxidation to oxidise the vicinal diol group formed in step a) to form an aldehyde group.

10 The polysaccharide starting material used in the process of the invention should preferably have the sialic acid unit at the reducing terminal end joined to the adjacent saccharide unit through its eight carbon atom. In step B) the 6, 7-diol group is oxidised to form an aldehyde at the carbon 7 atom.

15 In an alternative embodiment, where the sialic acid unit at the reducing terminal end is joined to the adjacent saccharide unit through the 9 carbon atom, in step B) a 7, 8 diol group is formed and is oxidised to form an aldehyde on the 8 carbon atom.

20 In the process of the invention it is preferred that the polysaccharide starting material has terminal saccharide unit at the non-reducing end which has a vicinal diol group and in which the starting material is subjected to a preliminary step, prior to step a), of selective oxidation to oxidise the vicinal diol group to an aldehyde, whereby in step a) the aldehyde is also reduced to form a hydroxy group which is not part of a vicinal diol group. The invention is of particular utility where the terminal unit of the reducing end of

25 the polysaccharide starting material is a sialic acid unit.

 According to a second aspect of the invention there is provided a new process in which a polysaccharide starting material having a terminal sialic acid at a non-reducing terminal end is subjected to the following steps.

- 30 c) a selective oxidation step to oxidise the non-reducing terminal sialic acid unit at the 7, 8 vicinal diol group to form a 7-aldehyde; and

d) a reduction step to reduce the 7-aldehyde group to the corresponding alcohol.

5 Generally this aspect of the invention is part of a process in which the polysaccharide starting material has a reducing terminal unit and is required to be subsequently conjugated to another molecule through that unit. In such a process the terminal unit is generally activated for instance by a reaction which would otherwise have activated a proportion of the sialic acid non-reducing terminal units. Such a reaction is, for instance selective oxidation of a vicinal diol moiety.

10 In the invention the polysaccharide may comprise units other than sialic acid in the molecule. For instance sialic acid units may alternate with other saccharide units. Preferably, however, the polysaccharide consists substantially only of units of sialic acid. Preferably these are joined 2 → 8 and/or 2 → 9.

15 Preferably the polysaccharide starting material has at least 2, more preferably at least 5, more preferably at least 10, for instance at least 50, saccharide units. For instance the polysaccharide may comprise at least 5 sialic acid units.

20 In a preliminary oxidation step the selective oxidation should preferably be carried out under conditions such that there is substantially no mid-chain cleavage of the polysaccharide chain ie. substantially no molecular weight reduction. Enzymes which are capable of carrying out this step may be used. Most conveniently the oxidation is a chemical oxidation. The reaction may be carried out with immobilised reagents such as polymer-based perrhuthenate. The most straight forward method is carried out with
25 dissolved reagents. The oxidant is suitably perrhuthenate, or, preferably, periodate. Oxidation may be carried out with periodate at a concentration in the range 1mM to 1M, at a pH in the range 3 to 10, a temperature in the range 0 to 60°C for a time in the range 1 min to 48 hours.

30 In the process, step a) is a step in which the sialic acid unit at the reducing end is reduced. Usually the unit at the reducing end of the

polysaccharide is in the form of a ketal ring and reduction in step a) opens the ring and reduces the ketone to an alcohol. The hydroxyl group at the 6-carbon atom is thus part of a vicinal diol moiety.

Suitable reduction conditions (from steps a) and d)) may utilise
5 hydrogen with catalysts or, preferably hydrides, such as borohydrides. These may be immobilised such as amberlite-supported borohydride. Preferably alkali metal hydrides such as sodium borohydride is used as the reducing agent, at a concentration in the range 1 μ M to 0.1M, a pH in the range 6.5 to 10, a temperature in the range 0 to 60°C and a period in the range 1 min to
10 48 hours. The reaction conditions are selected such that pendant carboxyl groups on the polysaccharide are not reduced. Where a preliminary oxidation step has been carried out, the aldehyde group generated is reduced to an alcohol group not part of a vicinal diol group. Other suitable reducing agents are cyanoborohydride under acidic conditions, e.g. polymer
15 supported cyanoborohydride or alkali metal cyanoborohydride, L-ascorbic acid, sodium metabisulphite, L-selectride, triacetoxyborohydride etc.

Between any preliminary oxidation step and reduction step a) and after step b) and between oxidation step c) and reduction step d) and between step d) and any subsequent oxidation step, the respective
20 intermediate must be isolated from oxidising and reducing agents, respectively, prior to being subjected to the subsequent step. Where the steps are carried out in solution phase, isolation may be by conventional techniques such as expending excess oxidising agent using ethylene glycol, dialysis of the polysaccharide and ultrafiltration to concentrate the aqueous
25 solution. The product mixture from the reduction step again may be separated by dialysis and ultrafiltration. It may be possible to devise reactions carried out on immobilised oxidising and reducing reagents rendering isolation of product straightforward.

The selective oxidation step, step b) is suitably carried out under
30 similar conditions to the preliminary oxidation step as described above.

Likewise the oxidation agent should be exhausted using ethylene glycol and the product recovered by suitable means such as dialysis and ultrafiltration.

5 The process of the first aspect of the invention and of the preferred embodiment of the second aspect which includes a subsequent oxidation step to activate the reducing terminal saccharide unit produces a polysaccharide derivative having a single reactive aldehyde moiety which is suitable for conjugating to amine-group containing substrates or hydrazine compounds. Preferably the conjugation reaction is as described in our earlier publications mentioned above, that is involving conjugation with an amine to form a Schiff
10 base, preferably followed by reduction to form a secondary amine moiety. The process is of particular value for derivatising proteins, of which the amine group is suitably the epsilon amine group of a lysine group or the N-terminal amino group. The process is of particular value for derivatising protein or peptide therapeutically active agents, such as cytokines, growth
15 hormones, enzymes, hormones, antibodies or fragments. Alternatively the process may be used to derivatise drug delivery systems, such as liposomes, for instance by reacting the aldehyde with an amine group of a liposome forming component. Other drug delivery systems are described in our earlier case US-A-5846951. Other materials that may be derivatised
20 include viruses, microbes, cells, including animal cells and synthetic polymers.

Alternatively the substrate may have a hydrazine group, in which case the product is a hydrazone. This may be reduced if desired, for additional stability, to an alkyl hydrazide.

25 In another preferred embodiment, oxidation step b) or a subsequent oxidation step after step d) is followed by the reaction of the aldehyde group with a linker compound, comprising an amine group and another functional group suitable for selective derivatisation of proteins or other therapeutically active compounds or drug delivery systems. Such a linker may, for instance,
30 comprise a compound having a functional group substituent for specific reaction with sulfhydryl groups and a di basic organic group joining the

amine group and the functional group. Reaction of the aldehyde moiety with the amino group forms a reactive conjugate suitable for binding to a substrate having a thiol (sulfhydryl) group. Such conjugates are of particular value for selective and site-directed derivatisation of proteins and peptides.

5 The derivatisation of proteins and drug delivery systems may result in increased half life, improved stability, reduced immunogenicity, and/or control of solubility and hence bioavailability and pharmaco-kinetic properties, or may enhance viscosity of solutions containing the derivatised active.

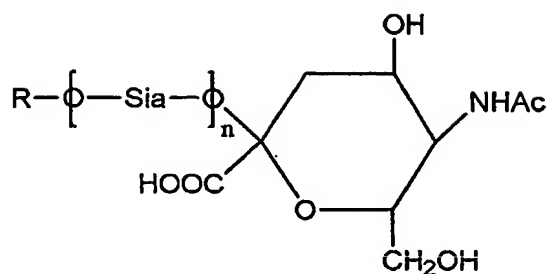
10 According to the invention there is also provided a novel compound which is an aldehyde derivative of a polysaccharide comprising sialic acid moieties, in which the terminal unit at the reducing end includes an aldehyde moiety and the terminal moiety at the non-reducing end comprises no aldehyde nor ketone moiety.

15 The novel compound may comprise saccharide units consisting only of sialic acid units or, alternatively, may comprise other saccharide units in addition to the sialic acid units. The compound may generally be as described above in relation to the first aspect of the invention.

20 The novel compound may be a polysialylated substrate, comprising at least one polysialic acid (polysaccharide) group conjugated on each molecule of substrate, the conjugation including a secondary amine, hydrazone or alkyl hydrazide linkage via the reducing terminal of the polysialic acid, and is substantially free of crosslinking via the non-reducing end of the polysialic acid group to another molecule of substrate. The
25 substrate may be, for instance, a biologically active compound, for instance a pharmaceutically active compound, especially a peptide or protein therapeutic, or a drug delivery system. Such actives are generally as described above.

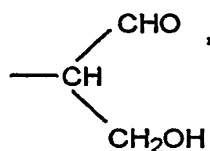
 The novel compound may have the general formula I

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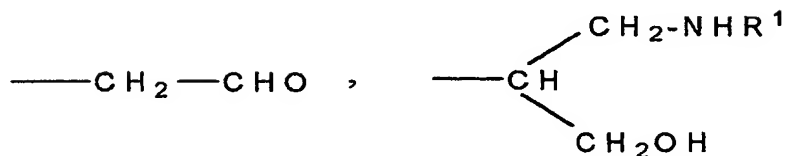


I

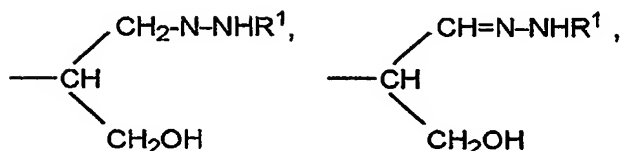
in which R is selected from



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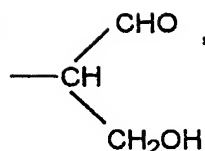
-CH₂CH₂NHR¹, CH₂CH=N-NHR¹ and CH₂CH₂NHNHR¹ in which R¹ is a polypeptide or a protein linked through the N terminal or a lysine and nitrogen atom, a drug delivery system or is an organic group having a functional substituent adapted for reaction with a sulfhydryl group;

20

n is at least 2; and

SiaO is a sialic acid unit.

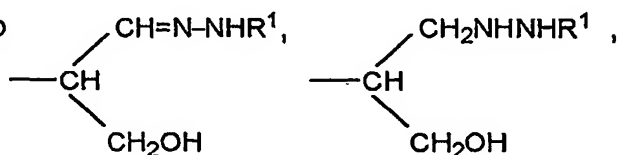
Where R is a group



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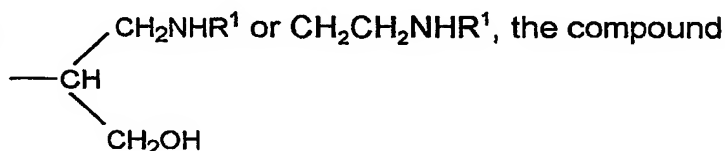
formula I is the polysialic acid derivative having an aldehyde group at the reducing terminal unit.

Where R is a group



- 5 $\text{CH}_2\text{CH=N-NHR}^1$ or $\text{CH}_2\text{CH}_2\text{NHNHR}^1$ the compound is a conjugate formed by reacting the aldehyde derivative of the polysialic acid with a hydrazide R^1NHNH_2 . A hydrazide is preferably an acyl hydrazide (R^1 has a terminal carbonyl group).

Where R is a group

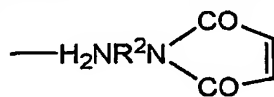


- 10 is a conjugate formed by reacting the aldehyde derivative of the polysialic acid with a primary amine group containing compound R^1NH_2 .

R^1 may be the residue of a peptide or protein therapeutic, for instance an antibody or fragment, an enzyme or other biologically active compound as described above. The group R^1 may comprise a linker moiety from the

- 15 active compound to the polysialic acid.

Alternatively, R^1 may be the residue of a linker reagent, for instance to form a derivatised polysialic acid suitable for conjugating to groups other than amine groups or hydrazides on active compounds. Examples are linker reagents of the formula



that is a N-maleimido

- 20 compound, in which R^2 is a dibasic organic group, for instance an aryene

oligo(alkoxy)alkane or, preferably, alkanediyl group, for instance a C₂₋₁₂-alkane diyl group.

The invention further provides compositions comprising the novel compounds and a diluent as well as pharmaceutical compositions
5 comprising novel compounds in which R¹ has biological activity, and a pharmaceutically acceptable excipient. Pharmaceutical compositions may be administered orally, intravenously, intraperitoneally, intramuscularly, subcutaneously, intranasal, intradermal or intratracheally.

There is provided in a second aspect of the invention a novel
10 compound which is the product of the process according to the second method aspect, namely the product of oxidation and reduction of a polysaccharide having a terminal sialic acid at the non-reducing end, the terminal unit of the novel compound having a 7-hydroxyl substituent.

The new method is of particular value for creation of a monofunctional
15 polysialic acid (PSA). It is based on an understanding of the tautomeric equilibrium of the reducing end ring of PSA's for instance colominic acid (CA) which is described in Fig 3. The reducing end sialic acid residue of PSA spontaneously forms an open ring ketone by tautomerisation (Fig. 3). In the dynamic equilibrium between ring and linear structures of the reducing
20 end sialic acid residue, the ketone moiety is present on only a subpopulation of PSA molecules at any one instant. As mentioned above however, it is here emphasized that the reactivity of the reducing end hemiketal is insufficient to be of practical utility for the attachment of PSA to proteins, which is why previously described methods do not employ this site on the
25 polymer for attachment to proteins or other drugs. Thus as illustrated in figure 3, in solution, the terminal sialic acid residue at the reducing end of polysialic acid exists in a tautomeric equilibrium. The ring-open form, although in low abundance in the equilibrium is weakly reactive with protein amine groups, and can give rise to covalent adducts with proteins in the
30 presence of sodium cyanoborohydride, although at a rate and to an extent that are of limited practical utility

In the invention, in order to achieve better defined products of protein conjugation with PSAs, we have now created a chemically modified form of polysialic acid that is monofunctional. The new form involves chemical modifications to both termini of the natural polysialic acid molecule. Unlike the original form of the reaction (Fig 1.), wherein the polymer becomes conjugated predominantly in the 2 to 8 orientation, with 'reducing end' outermost, the new form of the polymer becomes attached exclusively in the opposite orientation.

The new monofunctional form of the polysialic acid or other polysaccharide aldehyde to derivative is more conducive to the synthesis and manufacture of a pharmaceutically acceptable product, since it avoids the considerable complexity which is otherwise inadvertently created by use of polymer forms with unmodified reducing ends (Fig. 2). Production of the new form of the polymer (Fig 4) involves, selective oxidation, preferably by periodate as in our previous disclosures, to introduce an aldehyde function at the non-reducing end. Unlike the prior art illustrated in Fig. 1 however, this aldehyde moiety is then destroyed by reduction, for instance with borohydride. At the other end of the polymer, the borohydride reduction step also simultaneously locks open the ring structure of the reducing end, by reducing the hemiketal. This simultaneous reduction of the ketone to a hydroxyl moiety introduces a new diol functionality which is now amenable to selective oxidation in the second oxidation step. When the natural polymer has been (successively) oxidised with periodate, reduced with borohydride, and oxidised a second time with periodate, a new polymer form is created, which is truly monofunctional, having a single reactive group (an aldehyde) only at the reducing end (Fig. 4).

The protein reactivity (by reductive amination) of the various intermediates described in the 'double oxidation' process of figure 4 is described in Table 2. Notably, these data demonstrate that the intermediate 'CAOR', created by borohydride reduction of the periodate oxidised polymer, is inert towards protein targets, proving that both its aldehyde and hemiketal

moieties have been destroyed by borohydride reduction. In a second cycle of periodate oxidation of the 'protein inert' CAOR intermediate, a new polysialic acid derivative is created (CAORO) that is again reactive towards proteins (Table 2) and, moreover, is truly monofunctional in character, having a single aldehyde group at the 'reducing end' of the polymer, and being unreactive towards proteins at its other end. The monofunctional PSA can give rise only to single-orientation attachment to proteins, with the non-reducing end outermost, and is incapable of inadvertently cross-linking proteins (Fig. 5). This new scheme of reaction (Fig. 4), known as the 'double oxidation' method elegantly avoids the need to purify away the intended product from the various unintended products (described in Fig. 2), which are completely avoided in this new reaction scheme.

The invention is illustrated further in the accompanying examples.

Reference Example

Table 1. Yields of covalent PSA-protein conjugates generated by reductive amination with sodium cyanoborohydride using the natural form of polysialic acid (colominic acid, CA) from *E. coli*, via its weakly reactive reducing end. CA = colominic acid; CAO = oxidised colominic acid as in Fernandes *et al* 1996; Jain *et al* 2003. Sodium cyanoborohydride (NaCNBH₃) was used at a concentration of 4mg ml⁻¹.

The molar ratios in column 1 are the ratio of starting CA(O) to protein. n=3, \pm standard deviation.

Preparation	Degree of modification with CA molar ratio (CA:protein)
Catalase+CAO+NaCNBH ₃ (10:1)	0.77 \pm 0.16
Catalase+CAO+NaCNBH ₃ 50:1)	2.59 \pm 0.08
Catalase+CA+NaCNBH ₃ (50:1)	0.55 \pm 0.05
Catalase+CA (50:1)	0.65 \pm 0.04
Insulin+CAO+NaCNBH ₃ (25:1)	1.60 \pm 0.14
Insulin +CAO+NaCNBH ₃ (50:1)	1.65 \pm 0.14
Insulin +CAO+NaCNBH ₃ (100:1)	1.74 \pm 0.12

Insulin +CA+NaCNBH ₃ (25:1)	0.20±0.02
Insulin +CA+NaCNBH ₃ (50:1)	0.21±0.04
Insulin +CA+NaCNBH ₃ (100:1)	0.24±0.06

5

Examples

Materials

Ammonium carbonate, ethylene glycol, polyethylene glycol (8KDa),
 10 sodium cyanoborohydride (> 98% pure), sodium meta-periodate and
 molecular weight markers were obtained from Sigma Chemical Laboratory,
 UK. The colominic acid used, linear α -(2→8)-linked *E. coli* K1 polysialic
 acids (22KDa) was from Camida, Ireland., radioactive iodide (Na¹²⁵I) was
 purchased from Amersham, UK. Other materials included 2,4 dinitrophenyl
 15 hydrazine (Aldrich Chemical Company, UK), dialysis tubing (3.5KDa and
 10KDa cut off limits; Medicell International Limited, UK), Sepharose SP
 HiTrap, PD-10 columns (Pharmacia, UK), Tris-glycine polyacrylamide gels
 (4-20% and 16%), Tris-glycine sodium dodecylsulphate running buffer and
 loading buffer (Novex, UK). Deionised water was obtained from an Elgastat
 20 Option 4 water purification unit (Elga Limited, UK). All reagents used were of
 analytical grade. A plate reader (Dynex Technologies, UK) was used for
 spectrophotometric determinations in protein or CA assays. CD 1 outbred
 mice (8-9 weeks old; 29-35 g body weight) were purchased from Charles
 River (UK) and acclimatized for at least one week prior to their use.

25

Methods

Protein and colominic acid determination

Quantitative estimation of polysialic acids (as sialic acid) with the
 resorcinol reagent was carried out by the resorcinol method [Svennerholm
 30 1957] as described elsewhere [Gregoriadis et. al., 1993; Fernandes and
 Gregoriadis, 1996, 1997]. Fab (protein) was measured by the BCA
 colorimetric method [Brown et. al., 1989].

Example 1**Preparation of monofunctional polysialic acid :****1a Activation of colominic acid**

Freshly prepared 0.1 M sodium metaperiodate (NaIO_4) solution was
 5 mixed with CA (100mg CA/ml NaIO_4) at 20°C and the reaction mixture was
 stirred magnetically for 15 min in the dark. A two-fold volume of ethylene
 glycol was then added to the reaction mixture to expend excess NaIO_4 and
 the mixture left to stir at 20°C for a further 30 min. The oxidised colominic
 acid was dialysed (3.5KDa molecular weight cut off dialysis tubing)
 10 extensively (24 h) against a 0.01% ammonium carbonate buffer (pH 7.4) at
 4°C. Ultrafiltration (over molecular weight cut off 3.5kDa) was used to
 concentrate the CAO solution from the dialysis tubing. Following
 concentration to required volume, the filtrate was lyophilized and stored at
 -40°C until further use.

15 1b Reduction of colominic acid

Oxidised colominic acid (CAO; 22kDa) was reduced in presence of
 sodium borohydride. Freshly prepared 0.15mM sodium borohydride (NaBH_4 ;
 in 0.1M NaOH diluted to pH 8–8.5 by diluting with dilute H_2SO_4 solution) was
 mixed with CAO (100mg CA/ml) at 20°C and the reaction mixture was stirred
 20 for up to 2h in the dark. The pH was brought down to 7 by the completion of
 the reaction. The oxidised/reduced colominic acid (CAOR) was dialysed (3.5
 KDa molecular weight cut dialysis tubing) against 0.01% ammonium
 carbonate buffer pH (7) at 4°C. Ultracentrifugation was used to concentrate
 the CAOR solution from the dialysis tubing. The filtrate was lyophilized and
 25 stored at 4°C until further required. The determination of any aldehyde
 content was determined as described under 'determination of CA oxidation'.

1c Reoxidation of CA

After confirmation of no aldehyde content the oxidised/reduced
 colominic acid (CAOR) was again oxidised as reported under activation of
 30 colominic acid except CAOR was incubated with periodate solution for

longer time (up to 1h). The degree of oxidation in the CAORO product was measured on lyophilized powder obtained from this stage as well.

1d Determination of the oxidation state of CA and derivatives

Qualitative estimation of the degree of colominic acid oxidation was carried out with 2,4 dinitrophenylhydrazine (2,4-DNPH), which yields sparingly soluble 2,4 dinitrophenyl-hydrazones on interaction with carbonyl compounds. Non-oxidised (CA), oxidised (CAO), reduced (CAOR) and re-oxidised (CAORO) (5mg each), were added to the 2,4-DNPH reagent (1.0ml), the solutions were shaken and then allowed to stand at 37°C until a crystalline precipitate was observed [Shriner et. al., 1980]. The degree (quantitative) of CA oxidation was measured with a method [Park and Johnson, 1949] based on the reduction of ferricyanide ions in alkaline solution to ferric ferrocyanide (Persian blue), which is then measured at 630nm. In this instance, glucose was used as a standard.

1e Gel Permeation Chromatography

Colominic acid samples (CA, CAO, CAOR and CAORO) were dissolved in NaNO_3 (0.2M), CH_3CN (10%; 5mg/ml) and were chromatographed on over 2x GMPW_{XL} columns with detection by refractive index (GPC system: VE1121 GPC solvent pump, VE3580 RI detector and colation with Trisec 3 software, Viscotek Europe Ltd. Samples (5mg/ml) were filtered over 0.45µm nylon membrane and run at 0.7cm/min with 0.2M NaNO_3 and CH_3CN (10%) as the mobile phase.

Results

Colominic acid (CA), a polysialic acid, is a linear alpha-2,8-linked homopolymer of N-acetylneuraminic acid (Neu5Ac) residues (Fig. 1a). Periodate, however, is a powerful oxidizing agent and although selective [Fleury and Lange, 1932] for carbohydrates containing hydroxyl groups on adjacent carbon atoms, it can cause time-dependent cleavage to the internal Neu5Ac residues. Therefore, in the present work exposure of colominic acids to oxidation was limited to 15-60 min using 100 mM periodate at room temperature [Lifely et. al., 1981]. Moreover, as periodate decomposes on

exposure to light to produce more reactive species [Dyer, 1956], reaction mixtures were kept in the dark. The integrity of the internal alpha-2,8 linked Neu5Ac residues post periodate and borohydride treatment was analysed by gel permeation chromatography and the chromatographs obtained for the oxidised (CAO), oxidised reduced (CAOR), double oxidised (CAORO) materials were compared with that of native CA. It was found (fig. 6) that oxidized (15 minutes) (CAO) (6b), reduced (CAOR) (6c), double oxidised (1hr) (CAORO) (6d) and native(6a) CA exhibit almost identical elution profiles, with no evidence that the successive oxidation and reduction steps give rise to significant fragmentation of the polymer chain. The small peaks are indicative of buffer salts.

Quantitative measurement of the oxidation state of CA was performed by ferricyanide ion reduction in alkaline solution to ferrocyanide (Prussian Blue) [Park and Johnson, 1949] using glucose as a standard [results are shown in table 2]. Table 2 shows that the oxidized colominic acid was found to have a greater than stoichiometric (>100%) amount of reducing agent, i.e. 112 mol % of apparent aldehyde content comprising the combined reducing power of the reducing end hemiketal and the introduced aldehyde (at the other end). No reactivity was seen in CAOR demonstrating that the neutralisation of both the aldehyde and the hemi ketal of CAO had been successfully accomplished by borohydride reduction. After the second cycle of periodate oxidation, the aldehyde content of the polymer was restored to 95 % in CAORO (within experimental error of 100%) demonstrating the successful introduction of a new aldehyde moiety at the reducing end.

The results of quantitative assay of colominic acid intermediates in the double oxidation process using ferricyanide (Table 2) were consistent with the results of qualitative tests performed with 2,4 dinitrophenylhydrazine which gave a faint yellow precipitate with the native CA, and intense orange colour with the aldehyde containing forms of the polymer, resulting in an intense orange precipitate after ten minutes of reaction at room temperature.

Table 2

CA species	Degree of oxidation
colominic acid (CA)	16.1 \pm 0.63
colominic acid-oxidised (CAO)	112.03 \pm 4.97
colominic acid-reduced (CAOR)	0; Not detectable
colominic acid-oxidised-reduced-oxidised (CAORO)	95.47 \pm 7.11

Table 2: Degree of oxidation of various colominic acid intermediates in the double oxidation reaction scheme using glucose as a standard (100%, 1 mole of aldehyde per mole of glucose). $n=3 \pm$ s.d.

Example 2

Preparation of Fab-colominic acid conjugates

Fab was dissolved in 0.15 M PBS (pH 7.4) and covalently linked to different colominic acids (CA, CAO, CAOR and CAORO) via reductive amination in the presence of sodium cyanoborohydride (NaCNBH_3). Colominic acid from each step of the synthesis (starting material and products of each of Examples 1a to c) together with Fab in a CA:Fab molar ratios (100:1) were reacted in 0.15 M PBS (pH 7.4; 2ml) containing sodium cyanoborohydride (4mg/ml) in sealed vessels with magnetic stirring at $35 \pm 2^\circ\text{C}$ in an oven. The mixtures were then subjected to ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) precipitation by adding the salt slowly whilst continuously stirring, to achieve 70% w/v saturation. The samples, stirred for 1 h at 4°C , were centrifuged for 15 min (5000xg) and the pellets containing polysialylated Fab suspended in a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ and centrifuged again for 15 min (5000xg). The precipitates recovered were redissolved in 1 ml 0.15M Na phosphate buffer supplemented with 0.9% NaCl (pH 7.4; PBS) and dialysed extensively (24 h) at 4°C against the same PBS. The dialysates were then assayed for sialic acid and Fab content and the conjugation yield was expressed in terms of CA: Fab molar ratio. Controls included subjecting the native protein to the conjugation procedure in the presence of non-oxidised CA or in the absence of CA, under the

conditions described. Stirring was kept to a minimum to avoid concomitant denaturation of the protein. Polysialylated Fab was further characterised by size exclusion chromatography, ion exchange chromatography and SDS-PAGE.

5 **2b Ion exchange chromatography**

Zero (control) and 48 h samples (0.5 ml) from the reaction mixtures were subjected to ion exchange chromatography (IEC) on a Sepharose SP cation exchange column (1 ml; flow rate 1 ml/min; binding/washing buffer 50mM sodium phosphate, pH 4.0; elution buffer, 50mM sodium phosphate
10 buffer, pH 4.0 containing 1M sodium chloride). The columns were washed, eluted and the eluent fractions were assayed for CA and protein (Fab) content. PD-10 columns were used for desalting samples before applying to column.

2c SDS-Polyacrylamide gel electrophoresis

15 SDS-PAGE (MiniGel, Vertical Gel Unit, model VGT 1, power supply model Consort E132; VWR, UK) was employed to detect changes in the molecular size of Fab upon polysialylation. SDS-PAGE of Fab and its conjugates (CAOR and CAORO) of 0 (control) and 48 h samples from the reaction mixtures as well as a process control (non oxidised CA), was
20 carried out using a 4-20% polyacrylamide gel. The samples were calibrated against a wide range of molecular weight markers.

 In previous experiments [Jain et. al., 2003; Gregoriadis, 2001] with other proteins it was found that optimal CA:Fab (derived from sheep IgG) molar conjugation yields required a temperature of $35\pm 2^{\circ}\text{C}$ in 0.15 M PBS
25 buffer at pH 6-9 for 48h. The imine (Schiff base) species formed under these conditions between the polymer aldehyde and protein was successfully reduced with NaCNBH_3 to form a stable secondary amine [Fernandes and Gregoriadis, 1996; 1997]. Exposure of protein to periodate-oxidised natural CA generates a metastable Schiff's base CA-protein adduct
30 (as reported for the polysialylation of catalase [Fernandes and Gregoriadis, 1996]). Likewise, in the reaction of oxidised forms of CA with Fab, we first

created a metastable Schiff's base adduct, by incubation of the oxidised polymer with Fab for 48 h at 37°C which was then consolidated by selective reduction (reductive amination) with NaCNBH₃ (which reduces the Schiff's base imine structure, but not the aldehyde moiety of the polymer). In order to characterise the protein reactivity of the various CA intermediates of the 'double oxidation method' Fab was subjected to reductive amination in the presence of natural CA (CA), oxidized CA (CAO), oxidised-reduced CA (CAOR) and 'double oxidised' CA (CAORO). For these studies 22 KDa PSA was used, at CA:Fab molar ratio of (100:1). After 48h of incubation in the presence of NaCNBH₃, CA-Fab conjugates were isolated from reaction mixtures by precipitation with ammonium sulphate (as described in the "Examples") and the results expressed in terms of CA:Fab molar ratios in the resulting conjugates (Table 3).

CA species tested	Molar conjugation ratio (CA:Fab) attained
colominic acid (CA)	0.21:1 (weakly reactive)
colominic acid-oxidised (CAO)	2.81:1 (highly reactive)
colominic acid-oxidised-reduced (CAOR)	not detectable (reactivity destroyed)
colominic acid-oxidised-reduced-oxidised (CAORO)	2.50:1 (high reactivity regained)

Table 3: Synthesis Fab (protein) colominic acid compounds.

It is evident from Table 3 that when natural, non-oxidized CA (in the presence of cyanoborohydride) was used, a significant but low level of conjugation was observed (resulting in a 0.21:1, CA:Fab molar ratio) via reaction with the hemiacetal group of CA at its reducing end.

Formation of the CA-Fab conjugates was further confirmed by the co-precipitation of the two moieties on addition of $(\text{NH}_4)_2\text{SO}_4$ (CA as such does not precipitate in the presence of the salt). Evidence of conjugation was also confirmed by ion exchange chromatography (IEC, not shown) and polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 7).

For ion-exchange chromatography, polysialylated Fab obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation was redissolved in sodium phosphate buffer (50mM, pH 4.4) and subjected to IEC using a Sepharose SP HiTrap column (cation exchange). In contrast with results indicating complete resolution of CA (in the wash) and Fab (in eluted fractions), both CA and Fab from the 48h reaction samples co-eluted in the wash fractions, demonstrating the presence of CA-Fab conjugate.

Fig. 7 describes the analysis of the antibody Fab conjugates described above. These data confirm that the molecular weight distributions of the two conjugates are very similar (as expected, since the byproducts

obtained from the asymmetrically bifunctional CA make up only a small percentage of the total population of molecules). It is also evident from Fig. 7 that whether Fab conjugates were prepared from asymmetrically bifunctional CA (i.e. periodate oxidised natural CA) or from monofunctional PSA, that
5 conjugates of a wide molecular weight distribution, elevated from the molecular weight of underivatized Fab control, were created. This is consistent with the known polydispersity of the natural polymer reported in our previous published works. Fig. 7 also confirms that reductive amination with monofunctional CA gives rise to an Fab conjugate with comparable yield
10 to that of the earlier method based on periodate oxidised natural CA (described in Fig. 1). It is also evident from Fig 7 that only trace amounts of underivatized Fab remained in each conjugate sample. The trace amounts of remaining Fab were removed from these conjugates by ion exchange chromatography prior to *in vivo* studies (Example 3 below).

Example 3

In vivo studies

Samples of sheep IgG Fab fragment or conjugates with CAO or CAORO were radiolabelled with I^{125} as follows:

10% by volume of each of these samples was removed (~100 μ l) and
20 placed into fresh IODO-gen tubes. A 20 μ l sample of PBS containing 200mCi of I^{125} (as NaI) was added to the protein or conjugate and the tubes were capped and allowed to incubate at room temperature for 10 min. The contents of the tubes were then transferred to 500 μ l centrifugal filters (3.5kDa m. w. cut off) and the samples spun at 6,500 rpm in a
25 microcentrifuge. The eluent was discarded and the volume in the retentate (above the membrane) made up to 500 μ l. This process was repeated a further 5 times after which the radioactivity above (protein) and below (free iodine) the membrane for a 5 μ l sample was assessed using a Packard Cobra Gamma counter. If the counts due to free I^{125} were less than 5% of
30 those in the conjugated fraction, no further purification was carried out. If the

free I^{125} was >5% the purification cycle was repeated and the samples re-assessed.

CD1 mice (29-35 g body weight) were dosed with 40 μ g (100 μ l volume in PBS) of protein per mouse (~ 1.6 mg/kg) by the i.v. route (tail vein) as a single injection and 50 μ l samples of blood were then taken (using heparinised graduated capillaries) at time intervals from a different tail vein and added into 500 μ l PBS. The last bleed recorded was a total bleed in order to allow sufficient counts. Samples were then centrifuged at 3000 rpm for 10 minutes and recorded supernatant removed and placed in gamma counter tubes. Samples were counted along with representative samples of the injected protein in a Packard Cobra II auto gamma counter. Recorded counts were expressed as a percentage of the original dose injected.

Samples of radio-iodinated Fab, and CAO and CAORO Fab conjugates, and injected intravenously into mice to monitor half-life in the blood circulation. Figure 8 shows the pharmacokinetics of native Fab Vs Fab-colominic acid conjugates prepared by the original method (using CAO) and by the new double-oxidation method (using CAORO). These results demonstrate that CAO-Fab and CAORO-Fab gave rise to marked and significantly longer residence times in the circulation, than was the case for underivatised Fab, giving rise to increases of 6.28 fold and 5.28 fold (respectively) in AUC values compared to native Fab.

Example 4

Synthesis of maleimide conjugate

The CAORO synthesised in Example 1c above was reacted with 5 molar equivalents of N-[β -maleimidopropionic acid] hydrazide in 0.1M sodium acetate for 2h at 37°C. The product hydrazone was precipitated in ethanol, resuspended in sodium acetate and precipitated again in ethanol, redissolved in water and freeze-dried. The product is useful for site-specific conjugation to the thiol groups of cysteine moieties in proteins and peptides.

The monofunctional polysialic acid adlehyde derivative could also be reacted with a linking compound having a hydrazide moiety and a N-

maleimide moiety to form a stable hydrazone having an active maleimide functionality useful for reacting with a thiol group.

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CLAIMS

1. A process for producing an aldehyde derivative of a polysaccharide in which a polysaccharide starting material having a sialic acid unit at the reducing terminal is subjected to sequential steps of:
 - 5 a) reduction to reductively open the ring at the reducing terminal sialic acid unit, whereby a vicinal diol group is formed; and
 - b) selective oxidation to oxidise the vicinal diol group to form an aldehyde group.
- 10 2. A process according to claim 1 in which the sialic acid unit at the reducing terminal is joined to the adjacent saccharide unit through the 8 carbon atom whereby in step b) the 6,7 vicinal diol group is oxidised to form an aldehyde on the carbon-7 atom.
- 15 3. A process according to claim 1 or claim 2 in which the polysaccharide starting material has terminal saccharide unit at the non-reducing end which has a vicinal diol group and in which the starting material is subjected to a preliminary step, prior to step a), of selective oxidation to oxidise the vicinal diol group to an aldehyde, whereby in step a) the aldehyde is also reduced to form a hydroxy group which is not part of a vicinal diol group.
- 20 4. A process according to claim 3 in which the saccharide unit at the non-reducing end is a sialic acid unit.
5. A process according to claim 4 in which the polysaccharide is a polysialic acid consisting substantially only of units of sialic acid.
- 25 6. A process according to claim 4 or claim 5 in which the polysaccharide has at least 2, preferably at least 5 or more preferably at least 10, most preferably at least 50 sialic acid units in the molecule.
7. A process according to any of claims 2 to 6 in which a preliminary oxidation step is carried out under conditions such that there is substantially no mid-chain cleavage of the polysaccharide chain.
- 30 8. A process according to claim 7 in which the preliminary oxidation step is carried out in aqueous solution in the presence of periodate

at a concentration in the range 1mM to 1M, a pH in the range 3 to 10, a temperature in the range 0 to 60°C and a time in the range 1 min to 48 hours.

5 9. A process according to any preceding claim in which step a) is carried out under conditions such that pendent carboxyl groups on the polysaccharide are not reduced.

10 10. A process according to claim 9 in which step a) is carried out in aqueous solution in the presence of borohydride at a concentration in the range 1µM to 0.1M, a pH in the range 6.5 to 10, a temperature in the range 0 to 60°C and a period in the range 1 min to 48 h.

11. A process according to any preceding claim in which the aldehyde derivative of the polysaccharide is reacted with a substrate having a primary amine group or a hydrazide group.

15 12. A process according to claim 11 in which the product is reduced.

13. A process according to claim 11 or claim 12 in which the substrate is a peptide or a protein.

14. A process according to claim 13 in which the substrate is a peptide therapeutic.

20 15. A process according to claim 11 or claim 12 in which the substrate is a compound having a functional group substituent and a dibasic organic group joining the amine or hydrazide group and the functional group.

25 16. A process according to claim 15 in which the product is subsequently reacted with a compound having a thiol group, preferably a protein.

17. A process according to claim 11 or 12 in which the substrate is a drug delivery system, a cell, preferably a microbial cell or an animal cell, a virus or a synthetic polymer.

30 18. A compound which is an aldehyde derivative of a polysaccharide comprising sialic acid units, in which the terminal unit at the

reducing end includes an aldehyde moiety and the terminal unit at the non-reducing end comprises no aldehyde nor ketone unit.

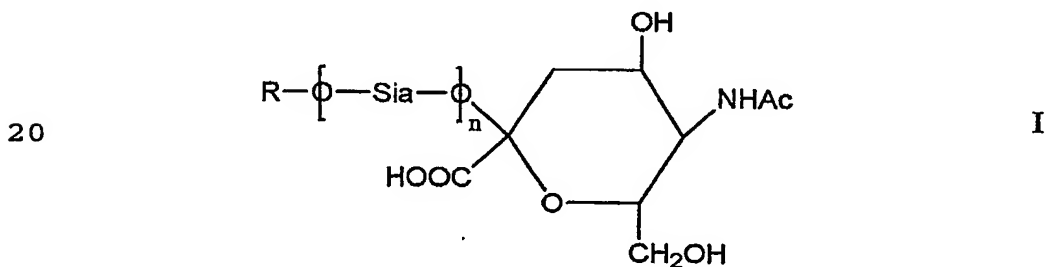
19. A compound which is a polysialylated substrate comprising at least one polysialic acid group conjugated on each molecule of substrate, the conjugation including a secondary amine a hydrazone or an alkyl hydrazide linkage via the reducing terminal of the polysialic acid.

20. A compound according to claim 18 which is substantially free of crosslinking via the non-reducing end of the polysialic acid group to another molecule or substrate.

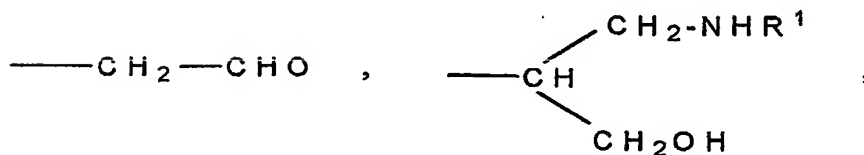
21. A compound according to any of claims 18 to 20 in which the sialic acid units are linked 2→8 and/or 2→9.

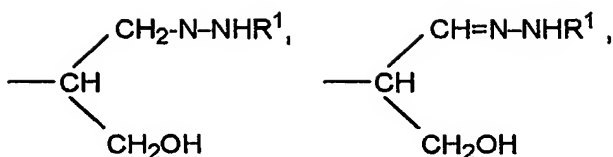
22. A compound according to any of claims 18 to 21 having at least 2, preferably at least 5, more preferably at least 10, most preferably at least 50, sialic acid units in polysaccharide chain.

23. A compound according to any of claim 18 to 22 having the general formula



25 in which R is selected from



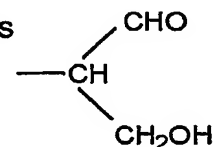


-CH₂CH₂NHR¹, CH₂CH=N-NHR¹ or CH₂CH₂NHNHR¹ in which R¹ is a polypeptide or a protein linked through the nitrogen atom of the N terminal or a lysine moiety, a drug delivery system a synthetic polymer, a cell or a virus or is an organic group having a functional substituent adapted for reaction with a sulfhydryl group;

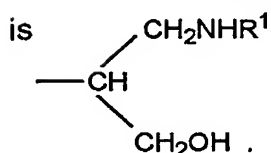
n is at least 2; and

SiaO is a sialic acid unit.

24. A compound according to claim 23 in which R is

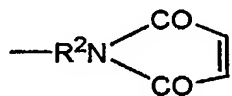


25. A compound according to claim 23 in which R



26. A compound according to claim 23 or claim 25 in which R¹ is a peptide or protein therapeutic, preferably an antibody or fragment.

27. A compound according to claim 25 in which R¹ is a group



12-alkanediyl group.

28. A composition comprising a compound according to any of claims 18 to 27 and a diluent.

29. A pharmaceutical composition comprising a compound according to claim 26 or claim 28 and a pharmaceutically acceptable excipient.

**ABSTRACT****Monofunctional Polysialic Acids for Protein Derivatisation
and Conjugation**

5

Derivatives are synthesised of polysaccharides having sialic acids at the reducing terminal end, in which the reducing terminal unit is transformed into an aldehyde group. Where the polysaccharide has a sialic acid unit at the non-reducing end it is passivated, for instance by converting into hydroxyl-substituted moiety. The derivatives may be reacted with substrates, for instance containing amine or hydrazine groups, to form non-cross-linked polysialylated compounds. The substrates may, for instance, be therapeutically useful peptides or proteins or drug delivery systems.

10

Figure 1a

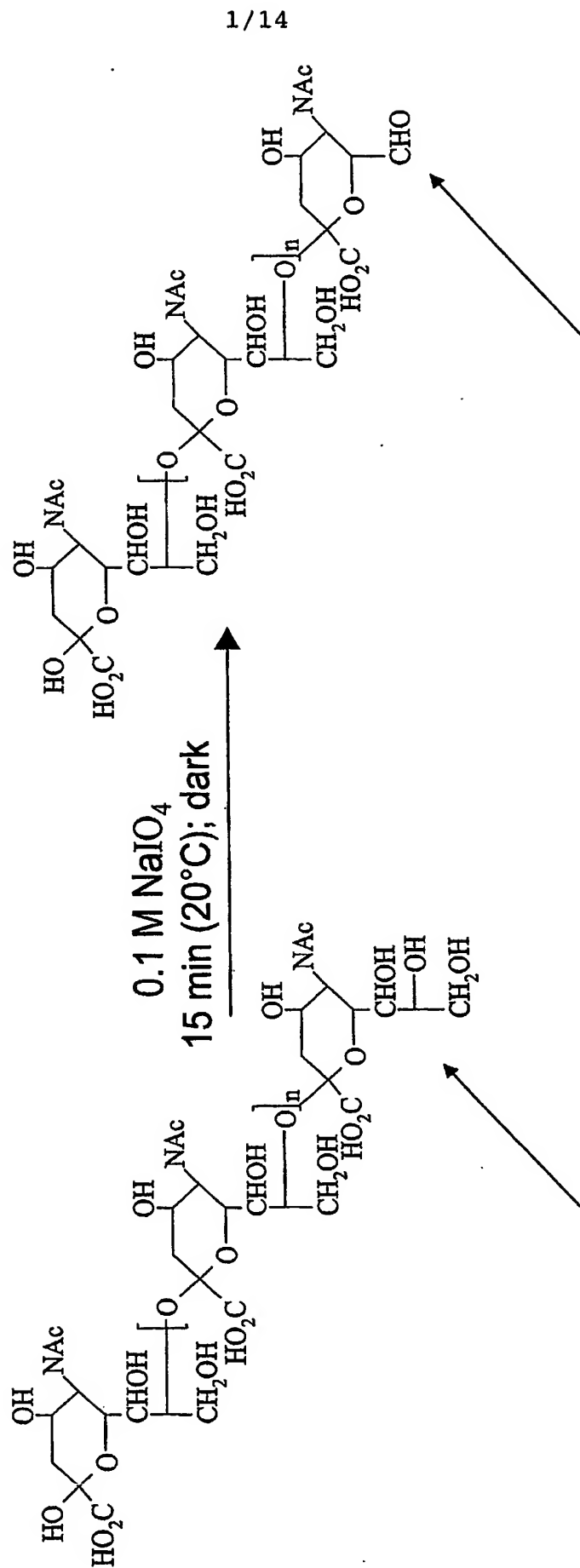


Figure 1b

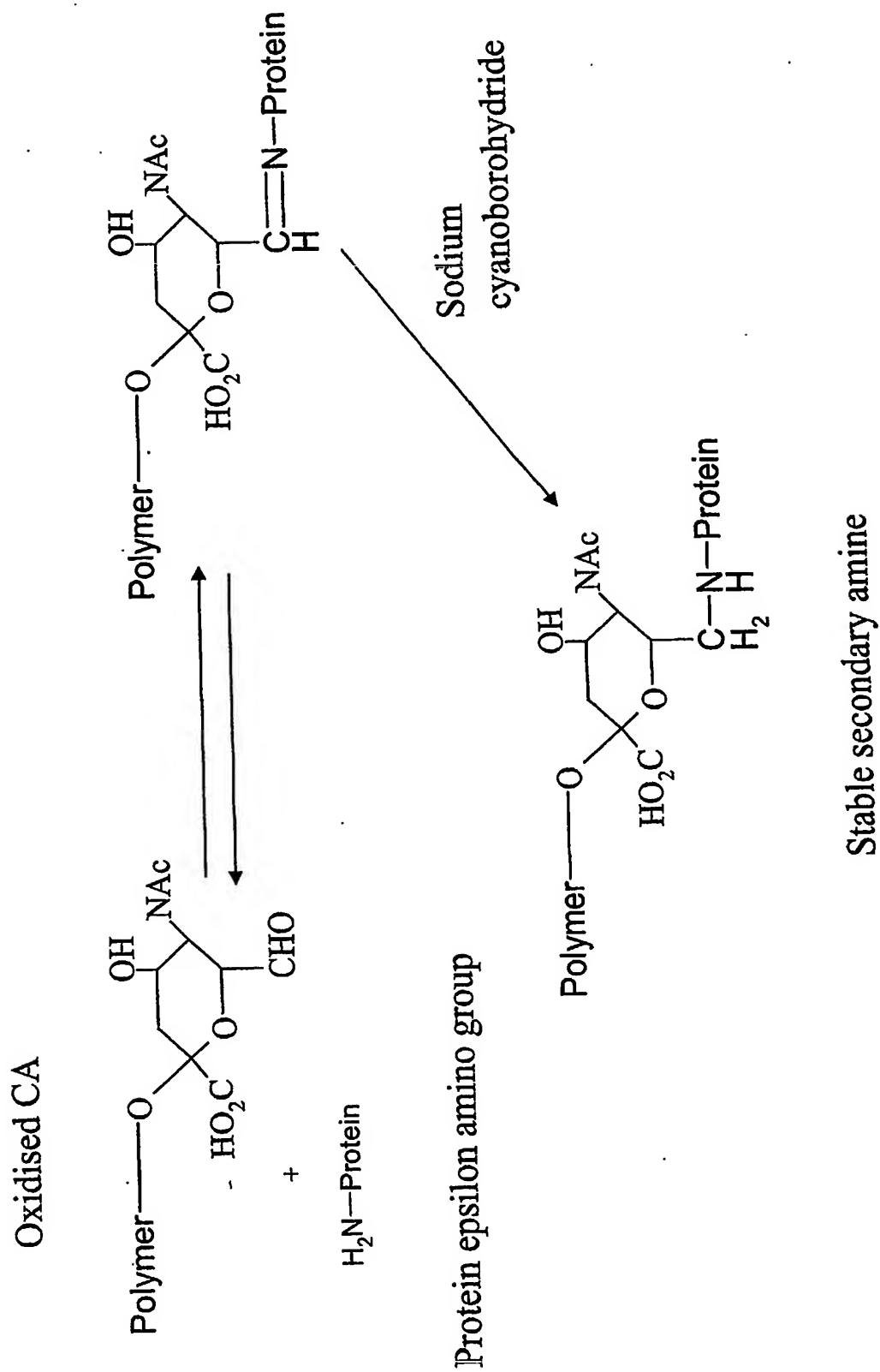


Figure 2a

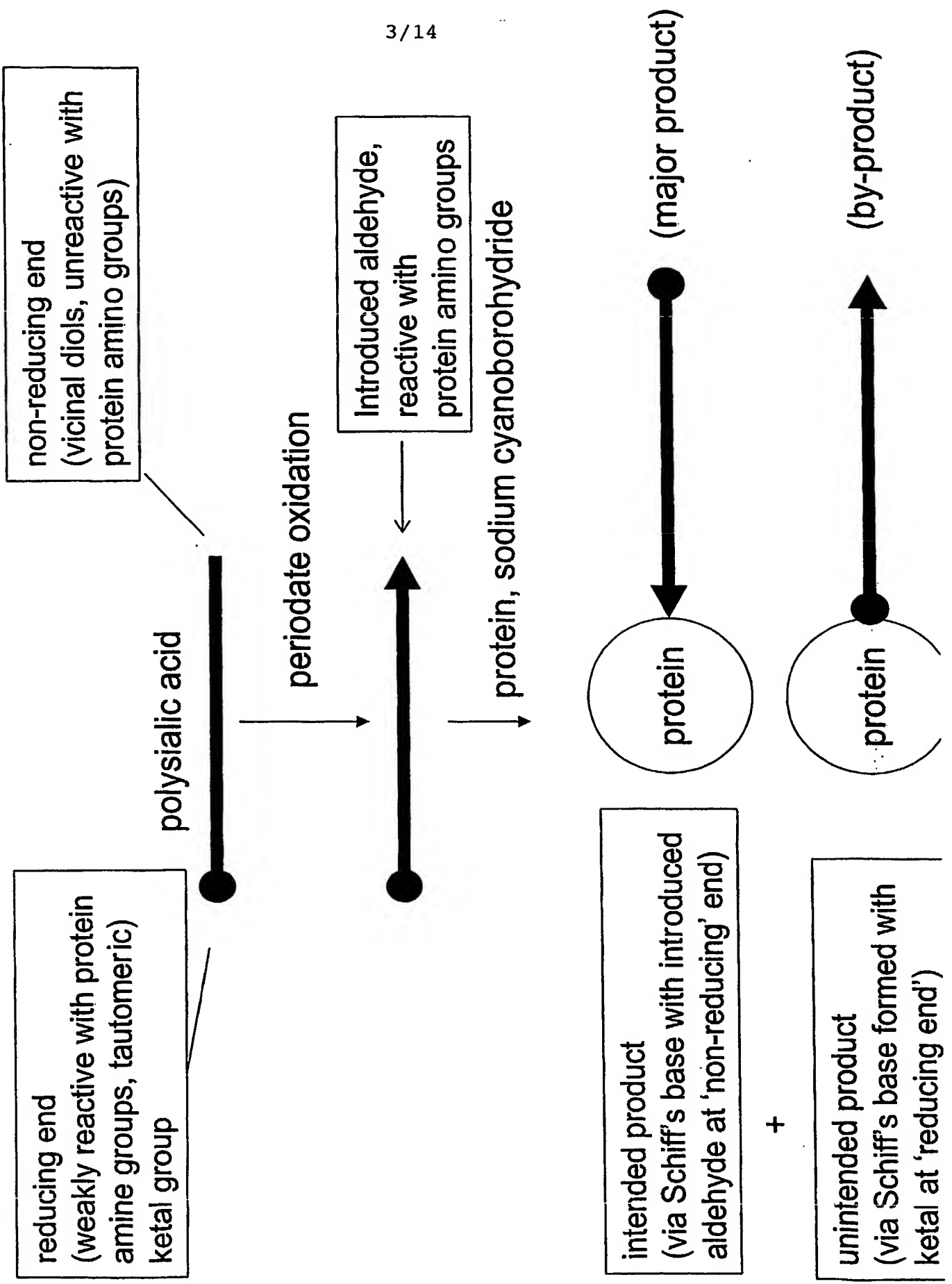


Figure 2b

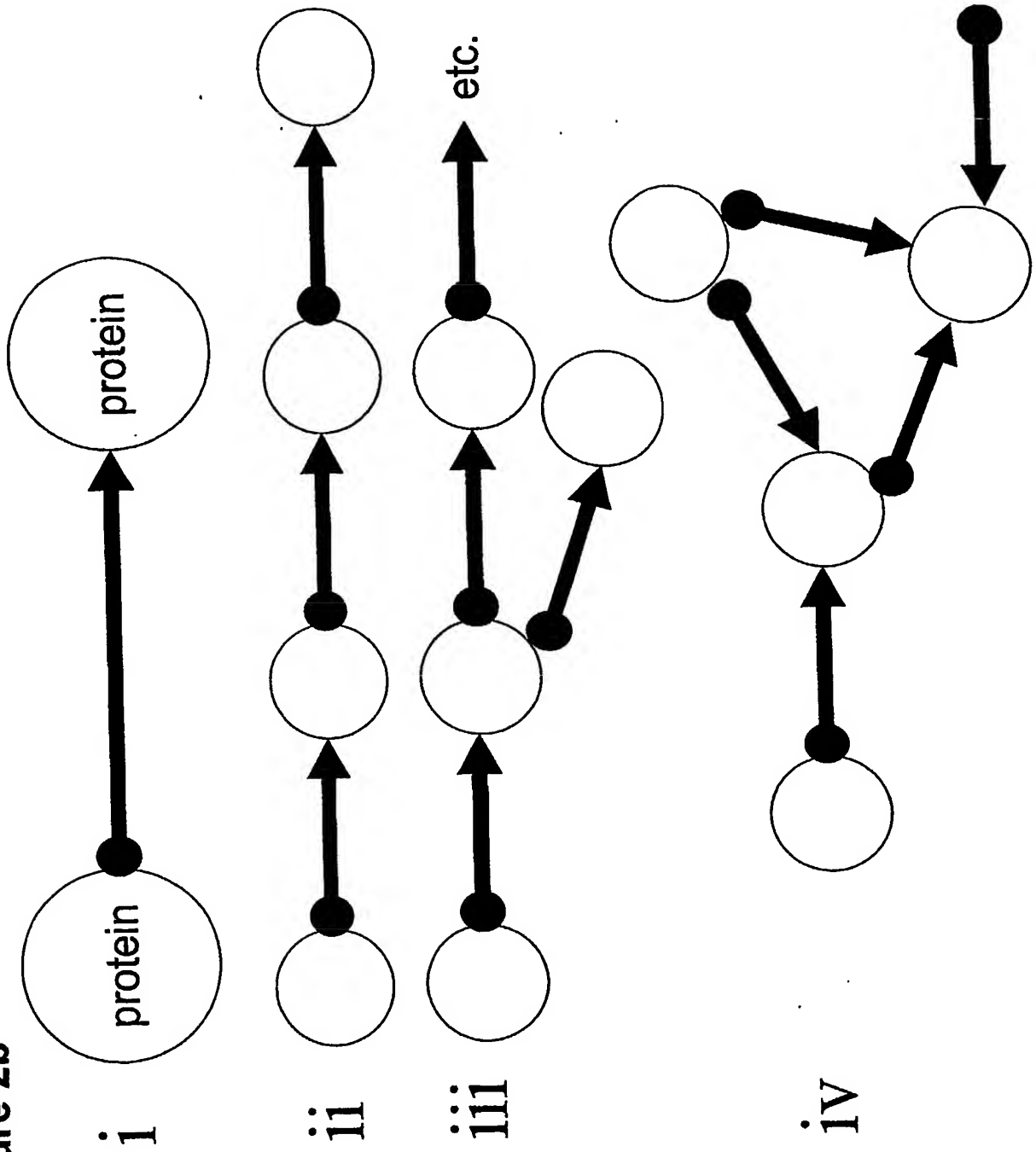


Figure 4a

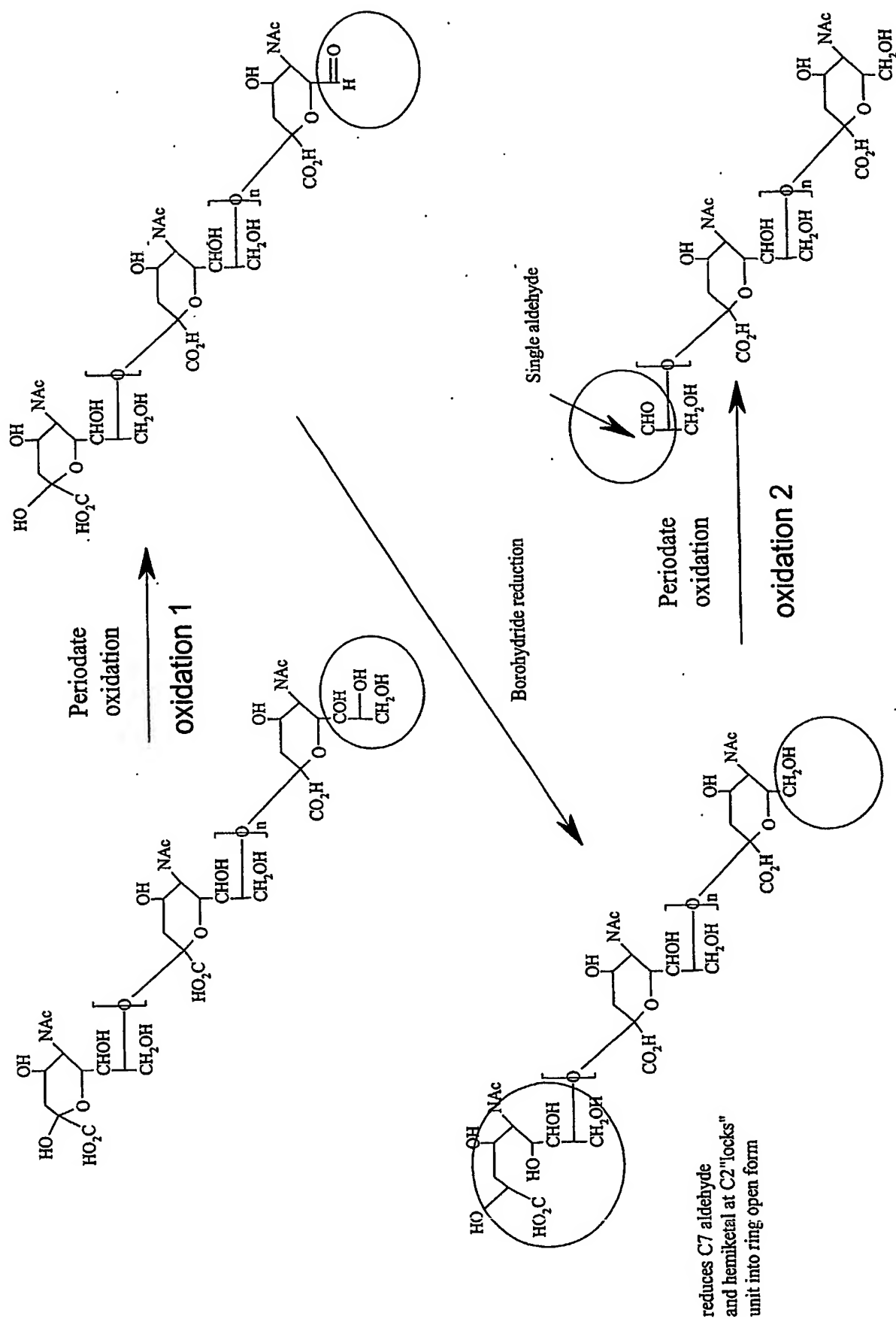


Figure 4b

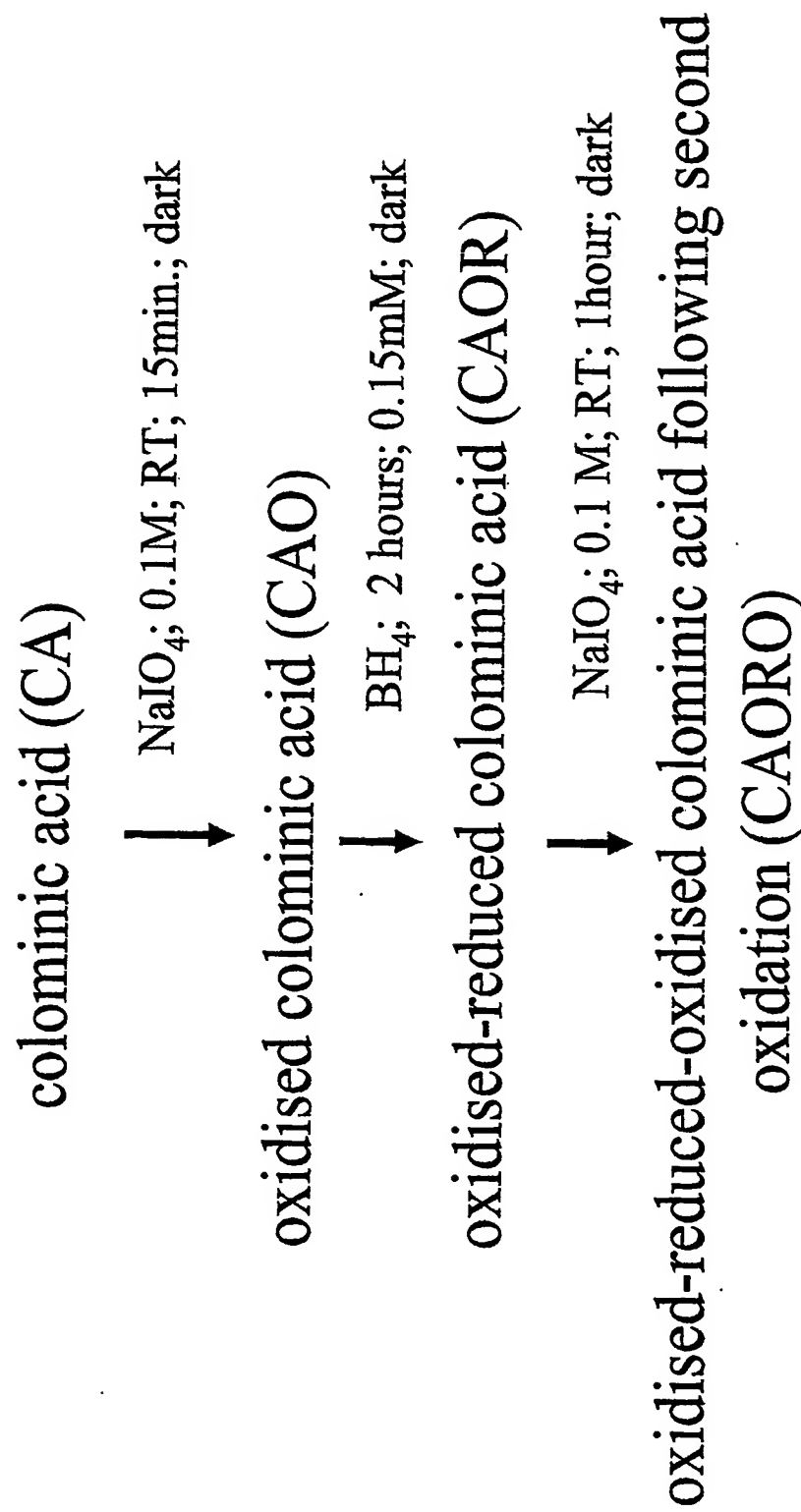
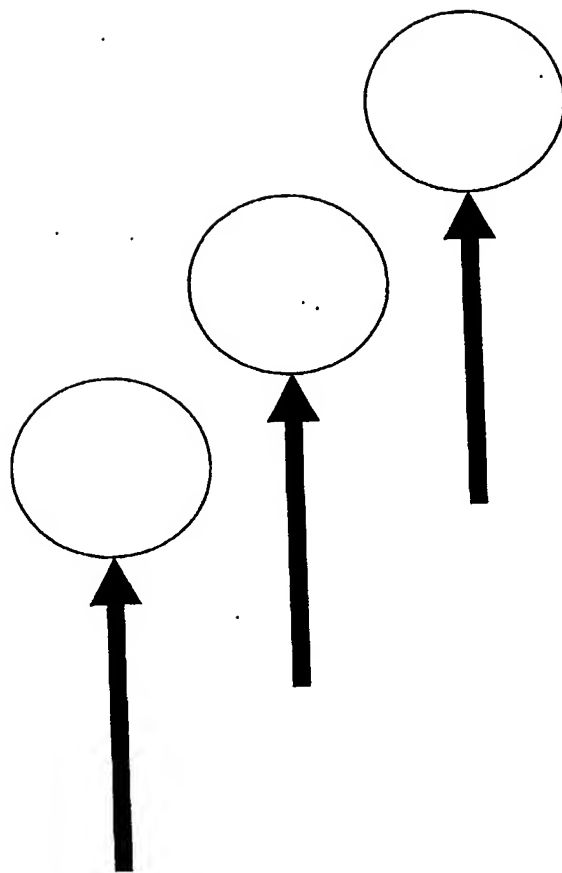


Figure 5



Monofunctional polysialic acid cannot form unintended by-products described for periodate-oxidised natural polysialic acid in Fig. 1

Figure 6a

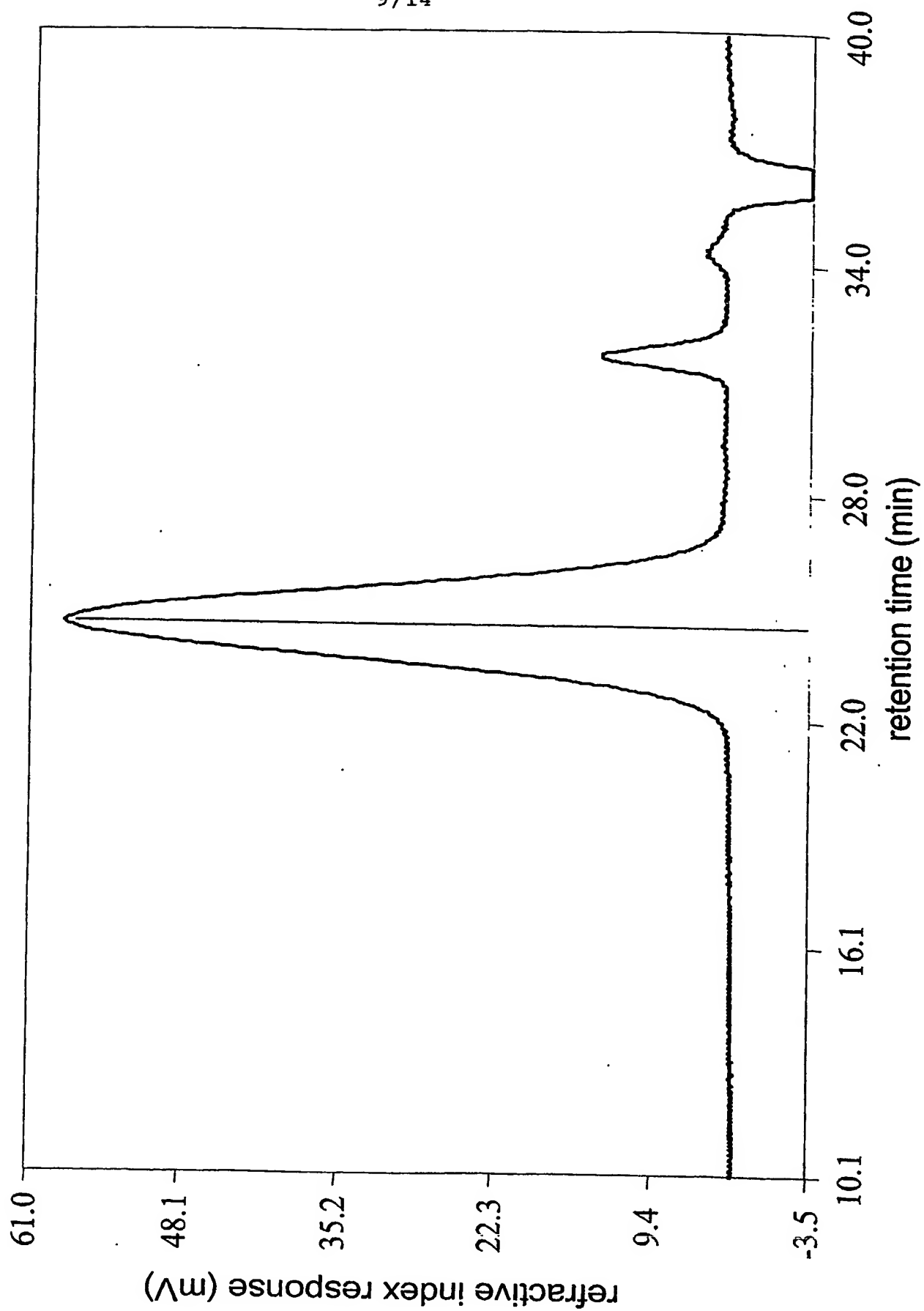


Figure 6b

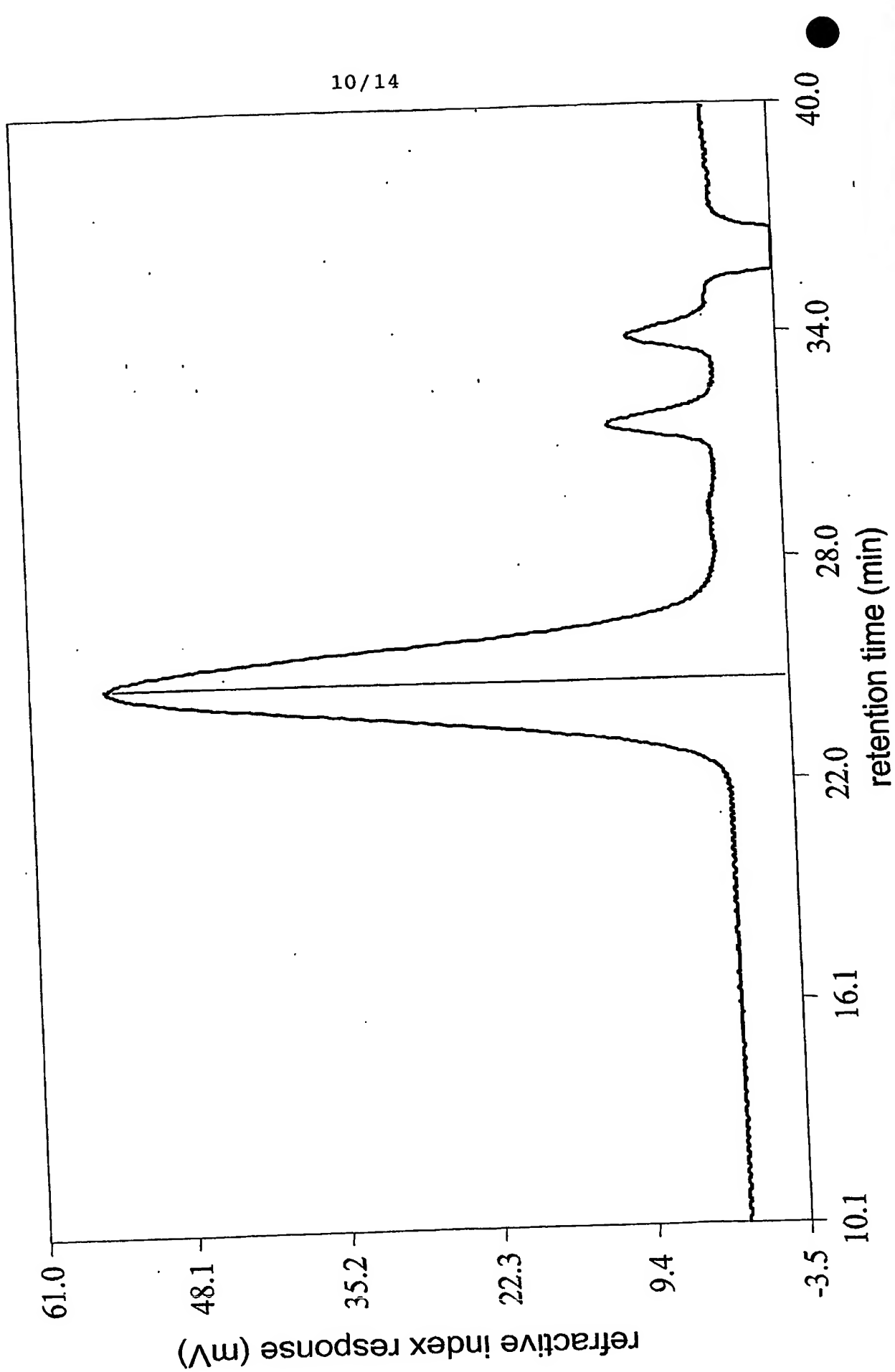


Figure 6c

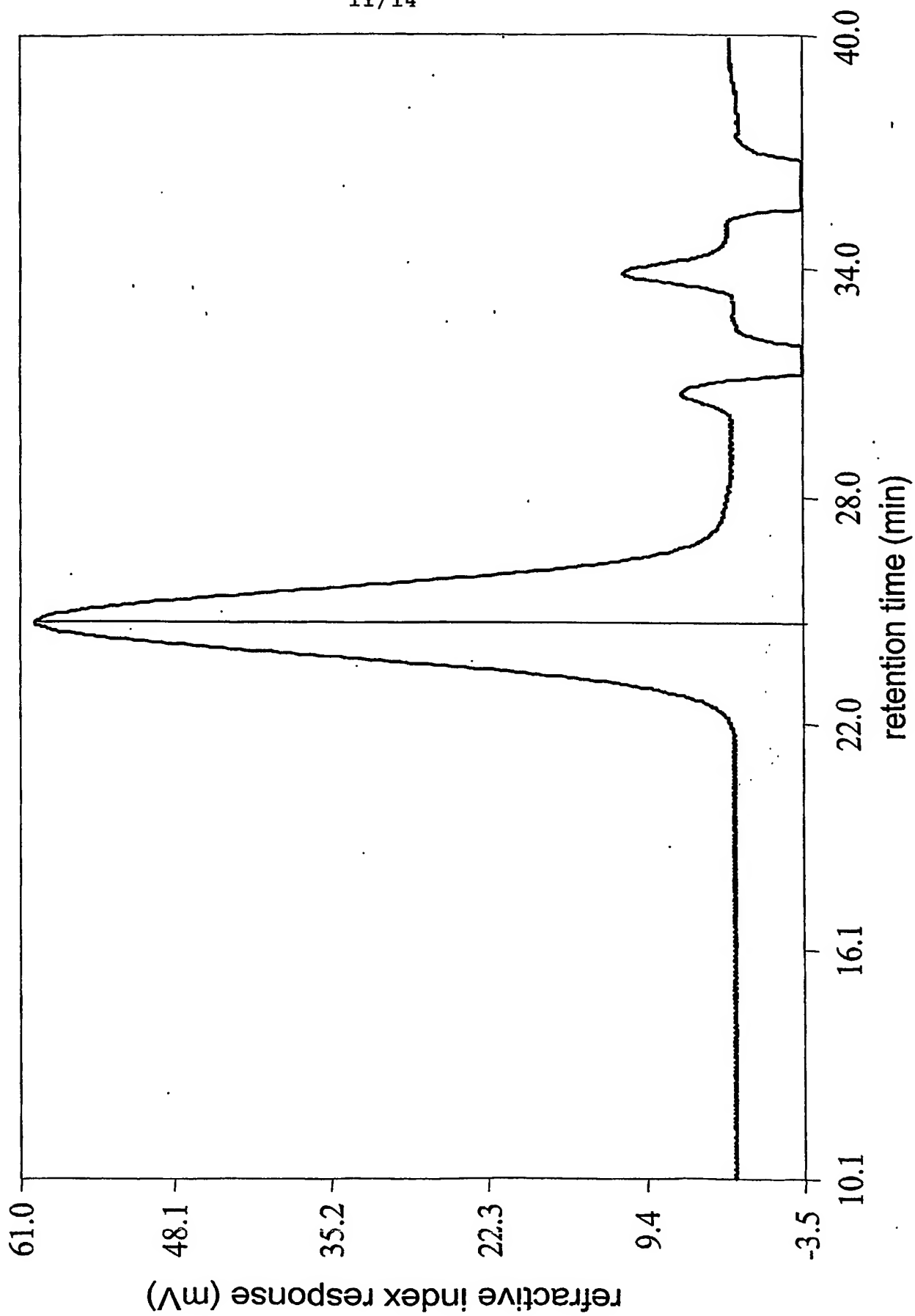
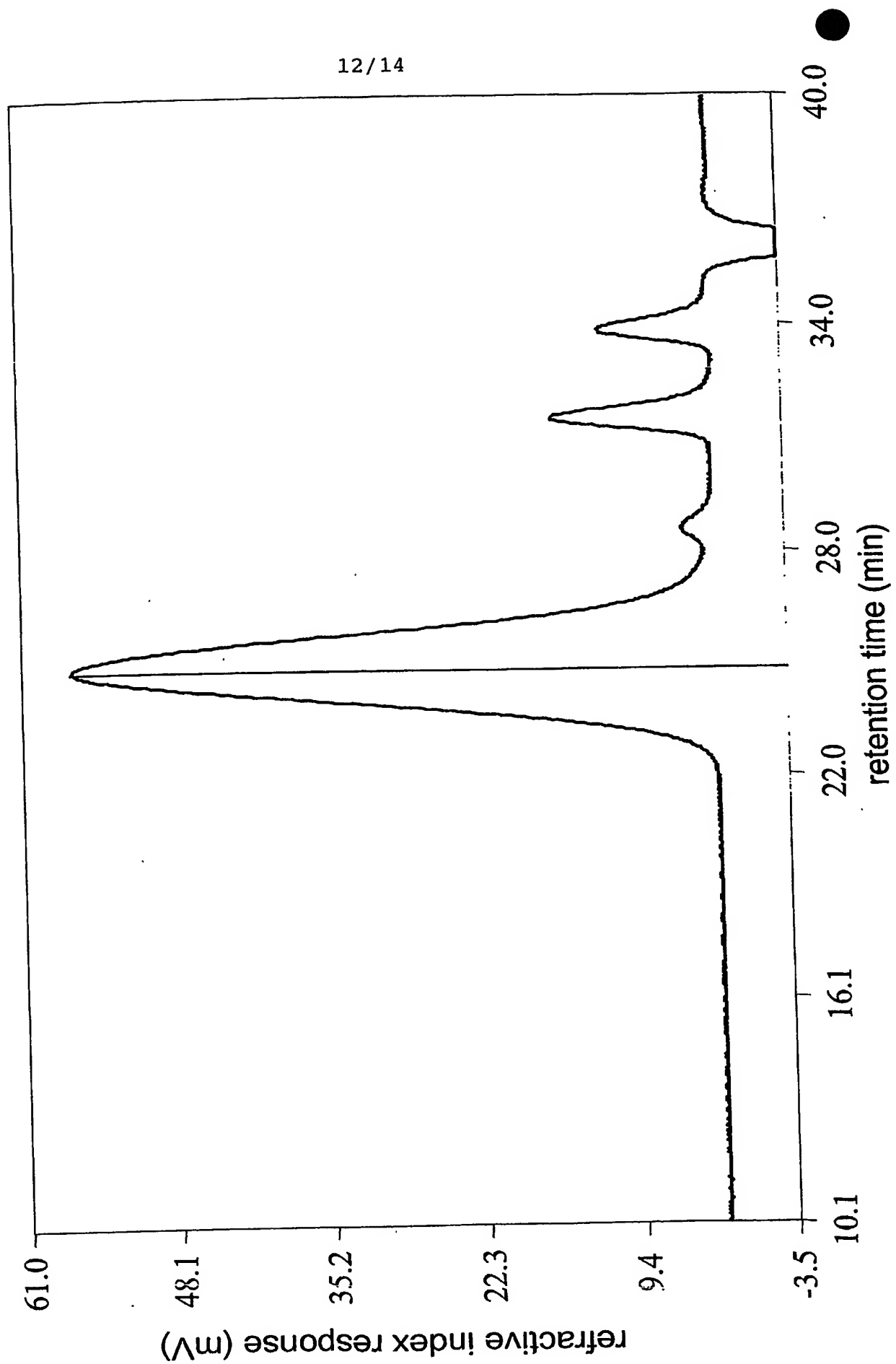


Figure 6d



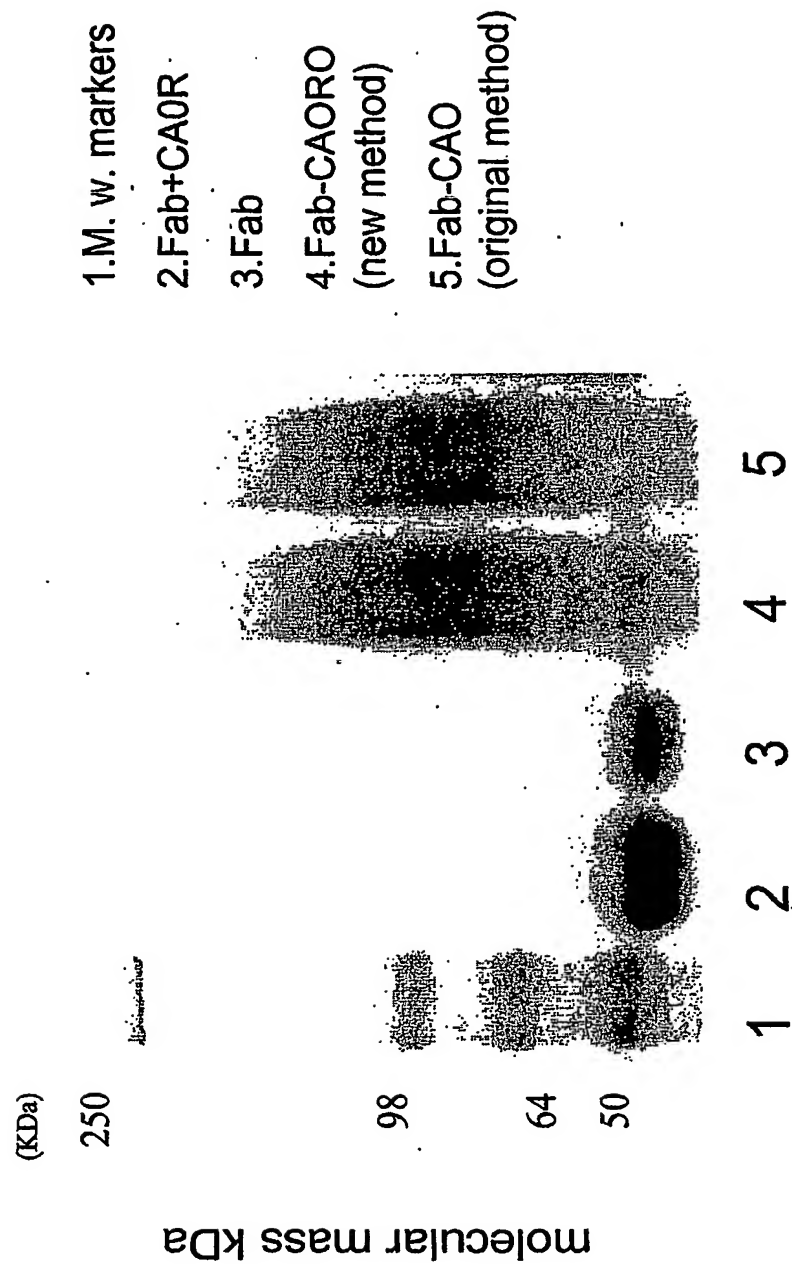
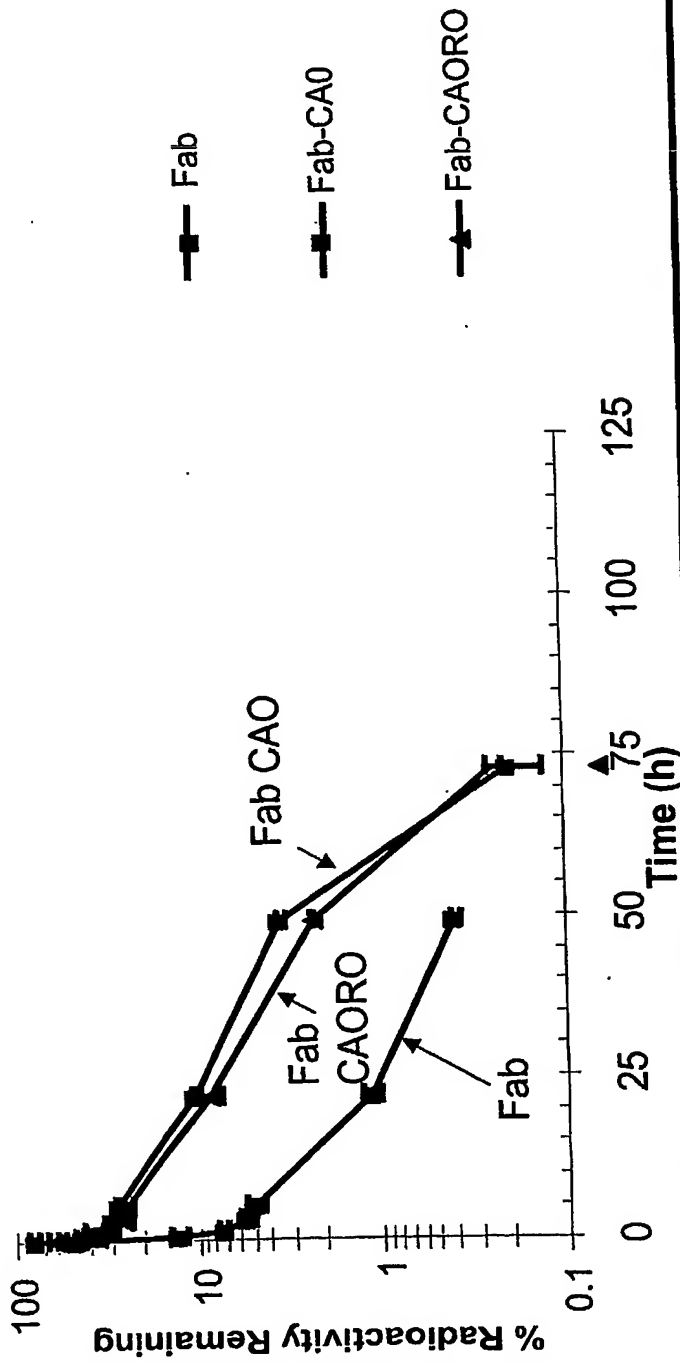


Figure 7

Figure 8



	Fab	Fab-conjugate prepared with CAO (original method)	Fab-conjugate prepared with CAORO (double oxidation method)
Area under curve	116	729	613

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